Influence of the amino acid moiety on deconjugation of bile acid amidates by cholylglycine hydrolase or human fecal cultures

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Abstract The influence of the chemical structure of the amino acid (or amino acid analogue) moiety of a number of synthetic cholyl amidates on deconjugation by cholylglycine hydrolase from *Clostridium perfringens* was studied in vitro at pH 5.4. Conjugates with alkyl homologues of glycine were hydrolyzed more slowly as the number of methylene units increased (cholylglycine > cholyl-β-alanine > cholyl-γ-aminobutyrate). In contrast, for conjugates with the alkyl homologues of taurine, cholylaminopropane sulfonate was hydrolyzed slightly faster than cholyltaurine, whereas cholylaminomethane sulfonate was hydrolyzed much more slowly. When glycine was replaced by other neutral α-amino acids, rates of hydrolysis decreased with increasing steric hindrance near the amide bond (cholyl-L-α-alanine >> cholyl-L-leucine >> cholyl-L-valine > cholyl-L-tyrosine >>> cholyl-D-valine). Conjugation with acidic or basic amino acids also greatly reduced the rates of hydrolysis, as cholyl-L-aspartate, cholyl-L-cysteate, cholyl-L-lysine, and cholyl-L-histidine were all hydrolyzed at a rate less than one-tenth that of cholylglycine. Methyl esterification of the carboxylic group of the amino acid moiety reduced the hydrolysis, but such substrates (cholylglycine methyl ester and cholyl-β-alanine methyl ester) were completely hydrolyzed after overnight incubation with excess of enzyme. In contrast, cholyl-cholamine was not hydrolyzed at all, suggesting that a negative charge at the end of the side chain is required for optimal hydrolysis. Despite the lack of specificity for the amino acid moiety, a bile salt moiety was required, as the cholylglycine hydrolase did not display general carboxypeptidase activity for other non-bile acid substrates containing a terminal amide bond: hippuryl-L-phenylalanine and hippuryl-L-arginine, as well as oleyltaurine and oleylglycine, were not hydrolyzed. Fecal bacterial cultures from healthy volunteers also hydrolyzed cholyl-L-valine and cholyl-D-valine more slowly than cholylglycine, suggesting that cholylglycine hydrolase from *Clostridium perfringens* has a substrate specificity similar to that of the deconjugating enzymes of the fecal flora. The results indicate that modification of the position of the amide bond, introduction of steric hindrance near the amide bond, or loss of a negative charge on the terminal group of the amino acid moiety of the bile acid conjugate greatly reduces the rate of bacterial deconjugation in vitro when compared to that of the naturally occurring glycine and taurine conjugates. — **Huijghebaert, S., and A. F. Hofmann.** Influence of the amino acid moiety on deconjugation of bile acid amidates by cholylglycine hydrolase or human fecal cultures. *J. Lipid Res.* 1986. **27:** 742-752.

Supplementary key words bacterial modification of bile acids • bile acids • bile salts

In mammals, the naturally occurring bile acids are C_{24} carboxylic acids which are formed from cholesterol in the liver. After their biosynthesis, their side chain is amidated with glycine or taurine to form N-acyl conjugates which are secreted into the bile and stored in the gallbladder (1, 2). During digestion, bile acids are secreted into the small intestine where they facilitate lipid absorption. The conjugated bile acids are not absorbed during digestion in the proximal small intestine because they are large, ionized molecules (3) that are resistant to deamidation by pancreatic and mucosal carboxypeptidases (4). Instead, the conjugated bile acids pass to the distal ileum, where they are efficiently absorbed by an active transport system. A small fraction of the bile acids escapes absorption from the ileum and is excreted in the feces.

Despite the resistance of the amide bond of conjugated bile acids to hydrolysis by tissue enzymes, the turnover rate of the glycine moiety of glycine-conjugated bile acids is several fold greater than that of the steroid moiety, indicating considerable deconjugation (by bacterial enzymes) and subsequent reconjugation (by hepatic enzymes) of the bile acids during enterohepatic circulation (5, 6). The bacteria mediating this deconjugation are considered to increase modestly in the more distal ileum and markedly in the colon, since unconjugated bile acids are not present in any appreciable proportion in jejunal content (7-9),

Abbreviations: CGH, cholylglycine hydrolase; UDCA, ursodeoxycholic acid; TLC, thin-layer chromatography; HPLC, high pressure liquid chromatography.

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but slowly increase proportionally in the ileum (8); in feces, bile acids are mostly in the unconjugated form (10–12). A number of bacteria strains with deconjugating activity have been isolated, such as enterococci, *Bacteroides,* anaerobic *Lactobacillaceae,* and *Clostridia* (reviewed in 13–15); and these have been shown to be present in ileal and fecal content (reviewed in 16).

Nair, Gordon, and Reback (17) isolated the bile acid deconjugating enzyme from *Clostridium perfringens,* and this enzyme, which is now available commercially, is termed cholylglycine hydrolase (CGH). Although these authors showed that the chemical structure of not only the side chain but also the steroid nucleus of bile acids influenced the rate of bile acid deconjugation by this enzyme, there has been little systematic examination of the effect of chemical structure of either the nuclear or the side chain moiety of conjugated bile acids on the rate of hydrolysis by this enzyme. Such information could be of interest since the taurine conjugate of ursodeoxycholic acid (UDCA) is now being explored as a therapeutic agent for cholesterol gallstone dissolution (18). Yet, when administered to healthy volunteers, this compound accumulates to only a limited extent in bile, probably because it is rapidly deconjugated during enterohepatic cycling (19) and the liberated UDCA is reconjugated in the liver, mostly with glycine (20).

In this report we have examined the effect of the chemical structure of the conjugating group on deconjugation in vitro by CGH or mixed anaerobic fecal cultures by using synthetically prepared cholyl conjugates amidated with a variety of amino acids as well as analogues of glycine or taurine. N-acyl amino acid conjugates in which the acyl group was not a bile acid but a long chain fatty acid were also examined. Finally, we tested whether CGH could hydrolyze the amide bond of C-terminal amino acids of small peptides. We reasoned that, since pancreatic carboxypeptidases have been shown to cleave bile acids conjugated with neutral or basic L-α-amino acids (4), it was possible that CGH might hydrolyze peptides.

MATERIALS AND METHODS

Bile acid conjugates

Fig. 1 shows the general structure of all cholyl conjugates used in this study. The chemical structures of the amino acids or amino acid analogues used for amidation of cholic acid are shown in **Table 1.** Cholyl conjugates were synthesized according to the method of Tserng, Hachey, and Klein (21) and were isolated and purified as has been recently reported (4, 22). The final products gave one spot on TLC using isoamyl acetate–propionic acid–n-propanol–water 4:3:2:1 (23) and were free of cholic acid. Substrate solutions (nominally 10 mM) were made

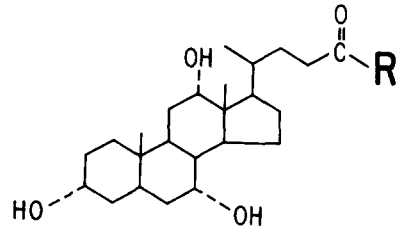

Fig. 1. General chemical structure of the cholyl conjugates. The chemical structure of the amino acids or amino acid analogues (= R) used for conjugation is given in Table 1.

in 0.05 N NaOH. For the bile acid conjugates used in the form of their methyl esters, 15% MeOH (by volume) was added to increase solubility. The bile acid concentration of the solutions was determined using a 3α-hydroxysteroid dehydrogenase assay (24); this procedure was necessary since many of the conjugates were extremely hygroscopic and thus difficult to weigh out accurately. Appropriate aliquots were taken to obtain the final concentration as indicated below.

Oleylglycine, the glycine amidate of oleic acid, and oleyltaurine were synthesized in the same manner as the cholyl amidates. Ricinoleylglycine and ricinoleyltaurine were generous gifts from Dr. Helmut Ammon, Medical College of Wisconsin, Milwaukee, WI.

Cholylglycine hydrolase (CGH)

Cholylglycine hydrolase (EC 3.5.1.24) was purchased from Sigma Chemical Company, St. Louis, MO (Product No. C-4018). The product is a partially purified lyophilized enzyme preparation from *Clostridium perfringens* (*welchii*). One unit (U) is defined as the amount of enzyme releasing 1 μmol of glycine from cholylglycine in 5 min at pH 5.6 and 37°C, determined under conditions as detailed by the manufacturer. Taurine should be released from cholyltaurine at one-third the rate found with cholylglycine.

The enzyme was dissolved to a concentration of 600 U per ml of H_2O containing 1 mM dithiothreitol. Aliquots were stored at −70°C until dilution for use in the enzyme assays. The rates of hydrolysis for cholylglycine remained within the same range during the duration of the experiments reported in this study.

Human fecal cultures

Samples of feces were collected immediately after defecation from three healthy volunteers ingesting a normal diet. Using an anaerobic glove box filled with nitrogen, a central portion of the feces was diluted with 25 parts (w/w) of 0.02 M phosphate buffer (pH 7.5) supplemented with 0.05% L-cysteine HCl. The suspension was homogenized, and coarse particles were allowed to settle. The supernatant was used within 2 hr of collection.

TABLE 1. Structural formulas of the amino acid moiety and chromatographic behavior of the cholyl conjugates on thin-layer chromatography (TLC) and reversed phase high pressure liquid chromatography (HPLC)

Classes of Conjugates	Substrates	Structural Formula of Amino Acid Moiety (= R)[a]	Adsorption Chromatography (TLC) R_f values[b]	Reverse-Phase Partition (HPLC) RRT[c]
Hepatic conjugates	C-glycine	$-NH-CH_2-COOH$	0.22	0.87
	C-taurine	$-NH-CH_2-CH_2-SO_3H$	0.06	0.54
Glycine homologue conjugates	C-β-alanine	$-NH-CH_2-CH_2-COOH$	0.30	1.08
	C-γ-aminobutyrate	$-NH-CH_2-CH_2-CH_2-COOH$	0.36	1.26
Taurine homologue conjugates	C-aminomethane sulfonate	$-NH-CH_2-SO_3H$	0.04	0.55
	C-aminopropane sulfonate	$-NH-CH_2-CH_2-CH_2-SO_3H$	0.08	0.54
Neutral L-α-amino acid conjugates	C-L-α-alanine	COOH $-NH-CH-CH_3$	0.28	1.02
	C-L-valine	COOH $-NH-CH-CH_2-(CH_3)_2$	0.40	1.63
	C-L-leucine	COOH $-NH-CH-CH_2-CH_2-(CH_3)_2$	0.47	2.23
	C-L-tyrosine	COOH $-NH-CH-CH_2-C_6H_4OH$	0.36	0.86
Neutral D-α-amino acid conjugates	C-D-valine	COOH $-NH-CH-CH_2-(CH_3)_2$	0.37	1.96
Acidic L-α-amino acid conjugates	C-L-aspartate	COOH $-NH-CH-CH_2-COOH$	0.19	0.64
	C-L-cysteate	COOH $-NH-CH-CH_2-SO_3H$	0.04	0.37
Basic L-α-amino acid conjugates	C-L-lysine	COOH $-NH-CH-CH_2-CH_2-CH_2-CH_2-\overset{+}{N}H_3$	0.04	0.71
	C-L-histidine	COOH $-NH-CH-CH_2-C=CH$ HN^{+} NH C H	0.04	0.68
	C-L-arginine[d]	COOH $\overset{+}{N}H_2$ $-NH-CH-CH_2-CH_2-CH_2-NH-C-NH_2$	0.03	0.71
Conjugates with non-acidic terminal group	C-glycine methyl ester	$-NH-CH_2-COO-CH_3$	0.35	1.41
	C-β-alanine methyl ester	$-NH-CH_2CH_2-COOCH_3$	0.33	1.52
		$-NH-CH_2CH_2-\overset{+}{N}(CH_3)_3$	0.02	1.43
N.B. Standards for TLC and HPLC				
Cholic acid			0.55	1.79
Ursodeoxycholyltaurine			0.10	0.47
Chenodeoxycholyltaurine			0.09	1.00

[a] For general structure of all cholyl conjugates, see Fig. 1. Sulfonate and carboxylates are shown in non-ionized form.
[b] TLC was performed on silica gel plates with the solvent system isoamyl acetate-propionic acid-n-propanol-water 4:3:2:1 (22).
[c] HPLC was performed on a reversed phase C_{18} column as described under Materials and Methods. Relative retention times of the cholyl conjugates were calculated, using the adjusted net retention volume of the cholyl conjugate versus the net retention volume of chenodeoxycholyltaurine.
[d] Cholylarginine was not used as a substrate in this study, but is included for completeness. It is hydrolyzed by CGH and by carboxypeptidase B (4).

Assay for deconjugation by CGH

The reaction mixture (1 ml) contained 2 mM cholyl conjugate, 5 mM sodium acetate buffer, pH 5.6, 1 mM EDTA, 10 mM mercaptoethanol, and 15 U of CGH. The components of the reaction mixture, except for CGH, were preincubated for 10 min at 37°C, and the reaction was then started by adding 15 U of CGH. Enzymes were added from a 1:4 dilution of the stock solution. (The volume containing 15 U was calculated from the supplier's statement of the enzyme's activity.) The reaction was carried out at pH 5.6, which is reported to be the optimal pH of the enzyme (17). A 250-μl sample was removed at each appropriate time interval and the reaction was stopped by adding 0.5 ml of 1 N NaOH. After centrifugation of the sample, the supernatant fluid was submitted to bile acid extraction.

Calculation of rates of deconjugation

Initial velocities were not always measurable in the linear range because determinations took place at concentrations below the K_m of rapidly hydrolyzed substrates. (The K_M of cholylglycine is 8 mM (17).) However, the use of 15 U enzyme and a 2 mM cholyl conjugate concentration allowed comparison of rates of hydrolysis for all of the substrates in parallel incubations. Accurate determination of the K_m and V_{max} was not possible for two reasons: first, the high pressure liquid chromatographic (HPLC) method (see below) used was not accurate during the initial hydrolysis since the method was based on a decrease in the concentration of conjugated bile acid rather than the appearance of unconjugated bile acid (see below); second, for several compounds, insufficient material was available to perform multiple measurements at high substrate concentration. Accordingly, results are expressed as μmol of cholyl conjugate hydrolyzed after 5 min.

Assay of deconjugation by fecal cultures

The rates of hydrolysis of cholylglycine, cholyl-L-valine, and cholyl-D-valine by human fecal bacteria under anaerobic conditions were compared in cultures incubated in parallel at 37°C. The culture broth was thioglycolate medium without added dextrose (BBL, Cockeysville, MD) or brain heart infusion broth (BBL) both supplemented with 0.5% yeast extract (Difco, Detroit, MI). After addition of 2 mM of cholyl conjugate, aliquots (2.7 ml) were dispensed into test tubes, sterilized at 120°C for 15 min, and preincubated in an anaerobic jar with an anaerobic hydrogen and carbon dioxide regenerating GasPak system (BBL). The cholyl conjugates were stable during autoclaving.

An aliquot, 0.3 ml, of fecal suspension, diluted 1:25 or 1:100 (v/v, with phosphate buffer at pH 7.5) was added to the bile acid medium in an anaerobic glovebox (Instruments for Research and Industry, Cheltenham, PA) under a N_2 stream. The cultures were incubated in the anaerobic jar with a H_2/CO_2 GasPak System and palladium catalyst at 37°C. Samples (500 μl) were removed at daily intervals under N_2, after transfer in the glovebox. (The N_2 gas had been purged of residual oxygen by passing it over an OxiClear gas purifier.) The reaction was stopped by adding 2.5 ml of 1 N NaOH; after removal of debris, the supernatant was submitted to bile acid extraction. Fecal incubations were performed at pH 7.5, a pH value within the range reported for cecal and fecal pH (25).

Extraction of bile acids

Conjugated and unconjugated bile acids were isolated using octadecylsilane cartridges (Bond Elut, Analytichem International, Harbor City, CA) as described (26). For qualitative screening of the isolated bile acids, thin-layer chromatography (TLC) was performed (23). For quantitation of the change in concentration of conjugated bile acids, which was used to assay hydrolysis, ursodeoxycholyltaurine or chenodeoxycholyltaurine (100 μmol) was added as internal standard before samples were analyzed using HPLC (27).

Thin-layer chromatography

TLC was used in screening studies aimed at estimating qualitatively the degree of hydrolysis of substrates in the presence of excess CGH or fecal cultures. The Bond Elut extracts were submitted to TLC on silica gel 60 plates in the solvent system isoamyl acetate–proprionic acid–n-propanol–water 4:3:2:1 (23). The plates were sprayed with phosphomolybdic acid and heated at 145°C until color appeared to estimate whether deconjugation had occurred as well as the pattern of bile acid biotransformation. For chromatograms of samples from fecal incubates, plates were also sprayed with a 2,4-dinitrophenylhydrazone spray in order to detect oxo (keto) bile acids. Cholic acid and metabolites were well separated from the cholyl conjugates (Table 1).

High pressure liquid chromatography

A Beckman 324 M high pressure liquid chromatograph was linked to a Hitachi variable wave length spectrophotometer, an Altex spectrophotometer flow cell (measurement at 200 nm) for detection, and an Altex electronic integrator (Model C-R 1A). The column was an Altex Ultrasphere-ODS (C_{18}) column, 25 cm × 4.6 cm, 5 μm particle size. The mobile phase was methanol–0.01 M monobasic potassium phosphate 3:1, adjusted with 4.2:1000 (vol/vol) 5 N NaOH and concentrated H_3PO_4 to pH 5.05–5.10 and filtered through a 0.45 μm filter. [This method is a modification of the method of Ruben and van Berge Henegouwen (27).] Absorbance at 200 nm was measured and the peak area was calculated by the integrator.

Relative retention times of cholyl conjugates were

calculated to the net retention time of chenodeoxycholyltaurine (see Table 1); the amount of remaining cholyl conjugate after an incubation interval was quantified from the peak areas related to those of known amounts of the respective cholyl conjugates. The amount of hydrolyzed cholyl conjugate was obtained by subtraction of the remaining amount from the initial amount in the enzyme "blank" and expressed as μmol of hydrolyzed conjugate or as percentage of original bile acid concentration in each sample. This indirect method of measurement was not accurate below 5% hydrolysis. It was assumed that each bile acid amidate had the same extinction coefficient at 200 nm as has been shown for the glycine and taurine amidates of the natural bile acids (28). Standard curves were linear over a range of 1 to 50 nmol. To show the accuracy of the method, known samples were analyzed by both HPLC and TLC enzymatic analyses. Ten percent of the extract was examined using TLC. After the chromatogram had been developed, the zones containing cholic acid were identified using an iodine chamber, scraped off the glass, and transferred to a test tube. A 3α-hydroxysteroid dehydrogenase assay (24) was used to determine the amount of cholic acid. A standard curve was made in the presence of similar amounts of silica gel. After centrifugation of the samples at 300 *g* for 10 min, the absorbance at 340 nm was determined by the supernatant. There was excellent agreement between the two values obtained in this manner and that obtained using HPLC. The TLC method, although more accurate in principle, was less satisfactory since fine particles of the adsorbent often remained suspended in solution and interfered with the assay; in addition, it was far more time-consuming.

Assay of the carboxypeptidase activity of cholylglycine hydrolase

To test whether CGH also possessed carboxypeptidase activity, that is, whether it could release terminal neutral or basic amino acids from peptide substrates, the hydrolysis of hippuryl-L-phenylalanine (Hip-Phe) and hippuryl-L-arginine (Hip-Arg) was assayed as described for carboxypeptidase A (29) and carboxypeptidase B (30). The latter enzymes remove, respectively, the neutral and basic L-α-amino acids from the C-terminal end of the peptides (31) and cholyl conjugates (4). Hydrolysis of Hip-Phe was measured spectrophotometrically at 254 nm (formation of hippuric acid) in 0.025 M Tris buffer, pH 7.5, in the presence of 0.5 M NaCl. Hydrolysis of Hip-Arg was measured similarly at pH 7.65 in the presence of 0.1 M NaCl. The reaction was started with 15 or 150 U of cholylglycine hydrolase; blanks containing carboxypeptidase A and B (Sigma), respectively, were included. These incubations were also performed in sodium acetate buffer (pH 5.6) containing EDTA and mercaptoethanol, as used for the assay of the cholyl conjugates.

Assay of hydrolase activity of cholylglycine hydrolase for non-bile acid amidates other than hippuryl conjugates

To test whether a bile acid nucleus is required for hydrolytic activity by CGH, hydrolysis of fatty acid (N-acyl) glycinates and taurates was examined. These substrates (2 mM) were incubated overnight in 5 mM sodium acetate buffer, pH 5.6, 1 mM EDTA, 10 mM mercaptoethanol, with 60 U of CGH. An aliquot (20 μl) was examined by TLC using benzene–acetone–acetic acid 50:50:1 as the developing solvent. For detection, plates were sprayed with phosphomolybdic acid (10 g/100 ml of ethanol) and heated at 145°C until color appearance.

RESULTS

Effect of the amino acid residue on the cholyl conjugate hydrolysis by cholylglycine hydrolase

Cholylglycine was hydrolyzed more rapidly than any other compound tested. Its initial rate of hydrolysis under the conditions of our assay was too rapid to be measured accurately, being complete after 2.5 min. By crude extrapolation, approximately 7 to 10 μmol of cholylglycine was hydrolyzed after 5 min.

Cholyl conjugates prepared with alkyl homologues of glycine (for structures, see Table 1) were hydrolyzed more slowly, and the rate of hydrolysis decreased with increasing number of methylene units, as illustrated in **Fig. 2.** Cholyl-β-alanine was hydrolyzed about one order of magnitude more slowly than cholylglycine. Cholyl-γ-aminobutyrate was hydrolyzed about two orders of magnitude more slowly than cholylglycine.

In contrast, an increase in the number of methylene units in the amino alkane sulfonic acid series caused an increase in rate of hydrolysis, as cholyl amino methane sulfonate was hydrolyzed quite slowly (0.27 μmol/5 min) compared to cholyl ethane sulfonate (taurine) which was hydrolyzed at a rate of 1.4 μmol/5 min. Cholyl-amino propane sulfonate was hydrolyzed still more rapidly (about 1.7 μmol/5 min, by extrapolation) (**Fig. 3**).

When the glycine moiety of cholylglycine was replaced by other neutral L-α-amino acids (**Fig. 4**), the rates of hydrolysis decreased from cholyl-L-α-alanine (2.3 μmol per 5 min after extrapolation) to cholyl-L-leucine (1.0 μmol per 5 min), cholyl-L-valine (0.35 μmol per 5 min), and cholyl-L-tyrosine (0.25 μmol per 5 min). Replacement of L-valine in cholyl-L-valine by D-valine virtually abolished deconjugation; only trace amounts of cholic acid (<5% of the initial amount) were measured after incubation for 30 min. When cholyl-D-valine (0.5 μmol substrate) was incubated overnight in the presence of excess enzyme (60 U), over 60% was hydrolyzed, indicating that this substrate could be hydrolyzed, albeit quite slowly.

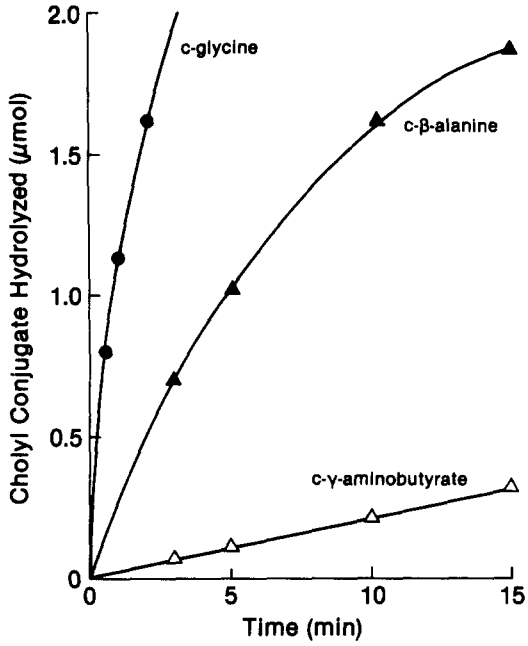

Fig. 2. Time course of hydrolysis of 2 μmol of cholyl (c) glycine (●) and its alkyl homologues, cholyl-β-alanine (▲) and cholyl-γ-aminobutyrate (△) by 15 U of cholylglycine hydrolase at pH 5.6. Each point is the mean of three determinations.

Introduction of a second acidic group, a carboxylic acid or sulfonic acid (Table 1) such as in cholyl-L-aspartate or cholyl-L-cysteate, caused a drastic reduction in hydrolysis rate (**Fig. 5**), with less than 0.5 μmol per 5 min hydrolyzed.

Cholyl conjugates with basic L-α-amino acids (cholyl-L-lysine and cholyl-L-histidine), which are present as zwitterions at the pH of the incubation, were hydrolyzed even more slowly than those with an acidic amino acid, with less than 0.2 μmol hydrolyzed after 5 min (**Fig. 6**).

Cholylglycine methyl ester and cholyl-β-alanine methyl ester were hydrolyzed extremely slowly: they were, however, completely deconjugated after overnight incubation with excess CGH (60 U). In contrast, cholylcholamine, which has a terminal positive charge, was not hydrolyzed at all.

Effect of some of the amino acid residues on cholyl conjugate hydrolysis by fecal cultures

To test whether the microflora in human feces had hydrolytic properties similar to those of the CGH from *Clostridium perfringens,* cholylglycine, cholyl-L-valine, and cholyl-D-valine were chosen as substrates for anaerobic incubation with fecal samples. The rate of hydrolysis of these three bile acid amidates varied widely: cholylglycine > cholyl-L-valine >> cholyl-D-valine. **Fig. 7** shows the time course of hydrolysis of the three cholyl conjugates by the fecal flora of a healthy volunteer, as measured by HPLC. Cholylglycine was hydrolyzed to 90% after 3 hr and completely cleaved after 8 hr of incubation. In contrast, hydrolysis of cholyl-L-valine became significant only after 1 to 2 days of incubation (18 and 40%, respectively) and reached only 65% after 4 days of incubation. The cholyl-D-valine concentration decreased still more slowly, with only 10% hydrolysis after 4 days of incubation.

TLC analyses of the samples, as well as those of other volunteers, showed similar courses of hydrolysis: the biotransformations are visualized in **Fig. 8.** All samples showed nearly complete hydrolysis of cholylglycine after 3 hr of anaerobic incubation. The major metabolite was unconjugated cholic acid. After 8 hr of incubation, cholic acid was partially metabolized to deoxycholic acid by 7α-dehydroxylation, which became the major metabolite in all of the samples after 1 day of incubation. In addition, as evidenced by the 2,4-dinitrophenyl hydrazone spray reagent, oxo (keto) bile acids were also produced by the microflora, presumably the action of bacterial hydroxysteroid dehydrogenases. Further characterization of these secondary bile acids was considered beyond the scope of this investigation.

Hydrolysis of cholyl-L-valine and cholyl-D-valine was clearly demonstrated using TLC after 2 and 4 days, respectively. The liberated bile acids occurred mainly as deoxycholic acid; cholic acid was present in trace amounts in most of the cultures and sometimes in slightly increased amounts after prolonged incubation with cholyl-L-valine for 4 days. Further, a metabolite (more polar than cholic acid, less polar than the cholyl conjugate) turned up in trace amounts after 2 days of incubation with cholyl-L-

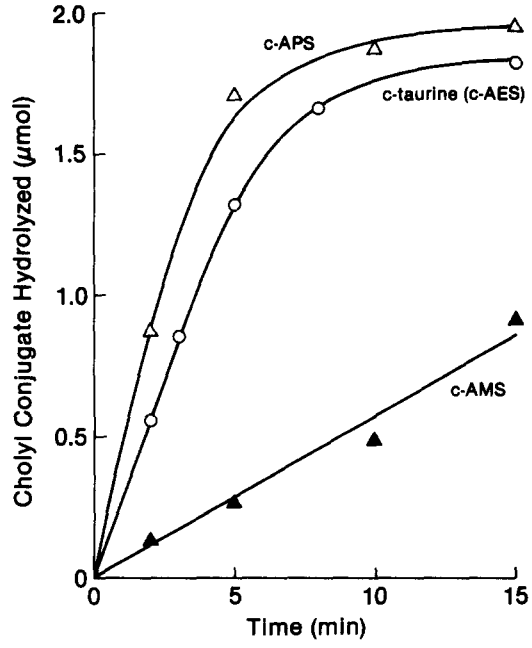

Fig. 3. Time course of hydrolysis of 2 μmol of cholyl (c) taurine (○) and its alkyl homologues, cholyl-aminomethane sulfonate (▲), C-AMS, and cholyl-aminopropane sulfonate (△), C-APS.

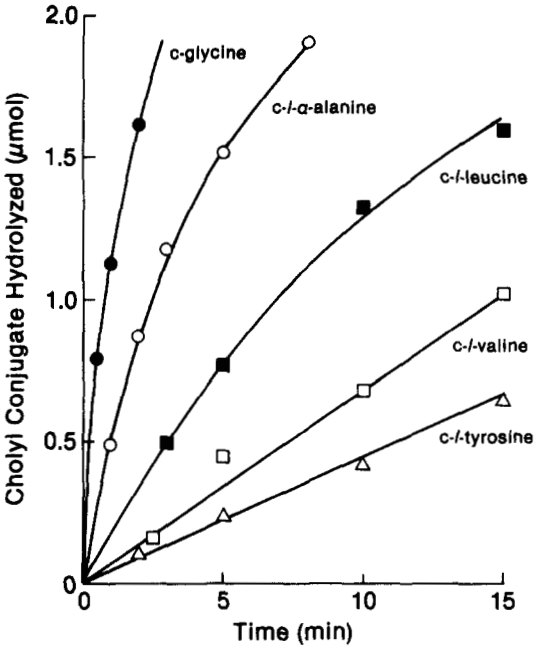

Fig. 4. Time course of hydrolysis of 2 μmol of cholyl (c) conjugate with neutral L-α-amino acids: cholylglycine (●); cholyl-L-α-alanine (○); cholyl-L-leucine (■); cholyl-L-valine (□); and cholyl-L-tyrosine (△) by 15 U of cholylglycine hydrolase at pH 5.6.

valine; from the shift in its R_f value, we deduced that it represented the corresponding 7α-dehydroxylated amidated form, that is deoxycholyl-L-valine. A trace metabolite with an analogous shift in R_f value was detected for cholyl-D-valine, probably representing deoxycholyl-D-valine. These latter results indicate that the intact cholyl conjugates may be 7-dehydroxylated by the intestinal microflora to a very small extent.

Assay of the carboxypeptidase activity of cholylglycine hydrolase

The CGH did not display carboxypeptidase activity at pH 7.5, as Hip-Phe or Hip-Arg were not hydrolyzed, even when the enzyme was present in excess. In contrast, the addition of carboxypeptidase A and B, respectively, caused an immediate rise in adsorbance at 254 nm indicating peptide bond hydrolysis. The experiments were also repeated in sodium acetate buffer, pH 5.6, in the presence of 2-mercaptoethanol and EDTA, since the pH, the reduced state of the enzyme, and metal chelation might influence the hydrolytic activity. However, no hydrolysis was detected.

Deamidation of non-bile acid aliphatic N-acyl amidates

No appreciable hydrolysis of oleylglycine, oleyltaurine, ricinoleylglycine, and ricinoleyltaurine was observed after overnight incubation with 60 U of CGH.

DISCUSSION

Hydrolysis of cholyl conjugates by cholylglycine hydrolase

The CGH from *Clostridium perfringens* was used as a test probe for intestinal bacterial deconjugation of cholyl amidate conjugates with amino acids or amino acid analogues other than glycine and taurine. Under the experimental conditions used, cholylglycine was deconjugated far more rapidly than any other bile acid amidate. Further, we demonstrated that not only the position of the amide bond, but also the charge, shape, and chiral nature of the amino acid or amino acid analogue influenced the rate of hydrolysis of the amide bond of the cholyl conjugate. In this respect, these experiments confirm and further extend the information on the structure–activity relationship of CGH, available from the early observations of Nair et al. (17) and the very recent report of Batta, Salen, and Shefer (32), which was published while this work was in progress. The latter report discusses the influence of the bile acid chain length, N-methylation of the amide bond, and introduction of oxo groups in the bile acid nucleus.

Our study suggests the following four requirements for optimal hydrolysis by CGH.

1. *The presence of a negative charge in the amino acid moiety.* The rate of hydrolysis was decreased by the addition of a second negative charge (cholyl-L-aspartate, cholyl-L-cysteate), but even more by the introduction of a positive charge, resulting in a zwitterionic bile acid conjugate (cholyl-L-lysine, cholyl-L-cysteate) or by methyl esterification of the terminal carboxylic acid (cholylglycine

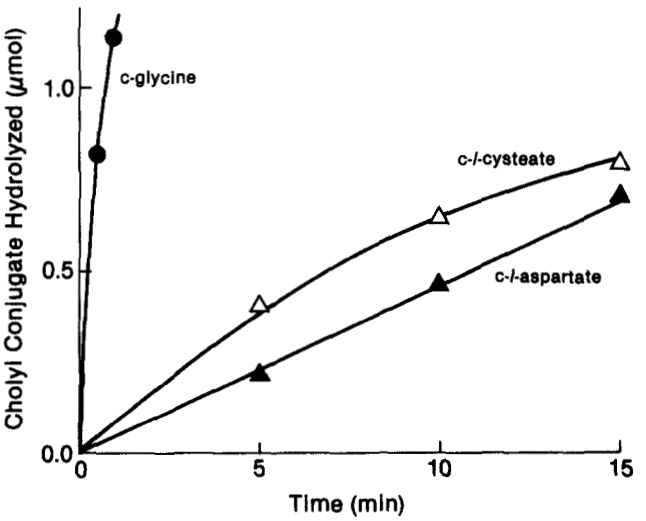

Fig. 5. Time course of hydrolysis of 2 μmol of cholyl (c) conjugates with acidic amino acids, cholyl-L-cysteate (△) and cholyl-L-aspartate (▲) by cholylglycine hydrolase at pH 5.6.

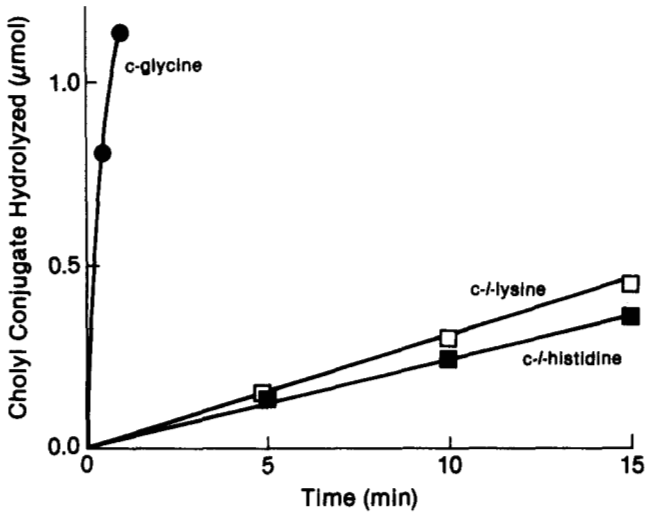

Fig. 6. Time course of hydrolysis of 2 μmol of cholyl (c) conjugates with basic amino acids, cholyl-L-lysine (□) and cholyl-L-histidine (■) by 15 U of cholylglycine hydrolase at pH 5.6.

methyl ester, cholyl-β-alanine methyl ester), which results in uncharged bile acid conjugates. Cholyl-cholamine, which is positively charged, was totally resistant to hydrolysis. These results suggest that the enzyme possesses a positively charged area for interaction with the carboxylic or sulfonic acid from the amino acid (or analogue) moiety, which allows optimal fitting of the substrate in the enzyme for hydrolysis. Our results are also consistent with the finding of Batta et al. (32) that the -$NHCH_2CH_3$ derivative of chenodeoxycholic acid is resistant to hydrolysis by CGH; this substrate does not possess a nucleophilic group for interaction with this cationic binding site.

2. *The presence of an optimal chain length between the position of the amide bond and the anionic group.* Increasing distance between the carboxyl group and the amide bond caused a progressive decrease in the rate of hydrolysis that was approximately 10-fold per methylene unit (cholylglycine > cholyl-β-alanine > cholyl-γ-aminobutyrate). This may explain why Nair et al. (17) did not notice any hydrolysis of cholyl-ε-aminovaleric acid (5 methylene units) by CGH, since the hydrolysis of this substrate after 5 min would be 1000 times less than that of cholylglycine. Conversely, at first sight, the increase of methylene units in the alkyl homologues of taurine caused an increase in hydrolysis by CGH. However, too few compounds were studied to permit such a conclusion about the structure–activity relationship, because the increase of hydrolysis may be limited to the amino-propane-sulfonate conjugate, whereas further addition of methylene units might decrease hydrolysis. Together the results suggest that the binding site for the negatively charged end of the side chain must be located at a fairly well-defined distance from the active center of the enzyme mediating the amide bond hydrolysis. As a longer or shorter alkyl homologue of glycine or taurine is used for conjugation, an increase or decrease in fitting of the substrate in the enzyme molecule will, respectively, enhance or reduce the rates of hydrolysis. However, the distance between the amide bond and the bile acid nucleus may also be important since C_{26} bile acids, which have a longer aliphatic side chain, and C_{23} bile acids, which have a shorter aliphatic side chain, are both hydrolyzed much more slowly than the naturally occurring C_{24} bile acids (32, 33, and unpublished observations from this laboratory).

3. *Absence of steric hindrance near the amide bond.* Reduced hydrolysis by CGH was observed for cholyl conjugates with a β-branched side chain (cholyl-L-valine) or a phenyl group (cholyl-L-tyrosine) in α-position, which is near the amide bond. Inspection of molecular models suggested that the steric hindrance due to the branched side chain in cholyl-valine was much greater for cholyl-D-valine than for cholyl-L-valine. With the D-valine amidate, the amide bond appears to be completely shielded by the branched side chain which might prevent its reaching the active center of the enzyme. With the L-valine amidate, the amide bond appears still accessible to the enzyme.

4. *The presence of a bile acid nucleus.* Despite its ability to remove a variety of amino acids from the respective cholyl conjugates, the CGH did not display carboxypeptidase activity against hippuryl-amino acid conjugates. Also, the taurine and glycine conjugates of oleic and rinoleic acid were not appreciably hydrolyzed, indicating that the enzyme possesses an affinity site for the bile acid nucleus.

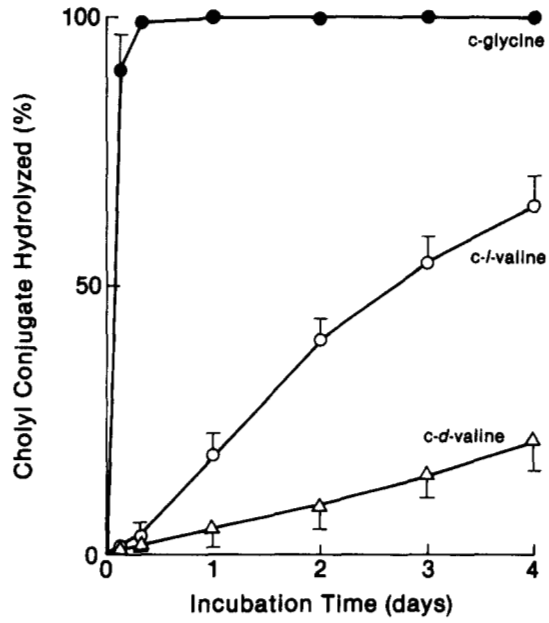

Fig. 7. Time course of hydrolysis of 1 mM of cholyl (c) glycine (●), cholyl-L-valine (○), and cholyl-D-valine (△) by cultures of human feces, diluted and anaerobically incubated as described under Materials and Methods. Values are obtained by HPLC (mean ± SD of three determinations).

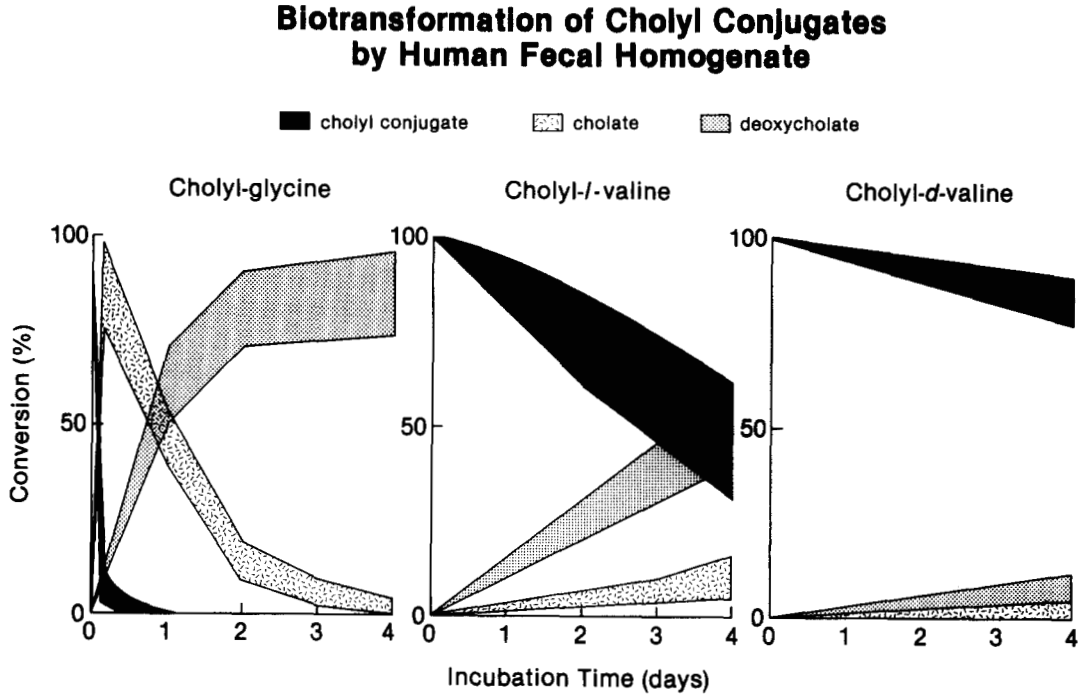

Fig. 8. Schematic representation of the time course of microbial hydrolysis of cholylglycine, cholyl-L-valine, and cholyl-D-valine (solid area) and the formation of the microbial metabolites, cholic acid and keto derivatives (dashed area) or deoxycholic acid and keto derivatives (stippled area) for each of the substrates at anaerobic incubation in fecal cultures from several volunteers, as estimated from TLC.

According to Nair et al. (17) as well as Batta et al. (32), substitution of a hydroxyl group by an oxo group in the bile acid nucleus reduces significantly the amount of hydrolysis, with complete resistance to hydrolysis being observed for 3,7,12-trioxo-5β-cholanoyl glycine (17). Thus, CGH is an N-hydroxy cholanoyl amino acid hydrolase and not a general N-acyl amino acid hydrolase.

Hydrolysis of the cholyl conjugates by fecal cultures

The results from the fecal cultures were in agreement with those from the incubation mixtures with CGH. They thus indicate that the specificity of the major deconjugating enzymes produced by a normal fecal flora is similar to that of CGH or, at least, that the deconjugating enzymes of a variety of bacterial species share the high affinity for cholylglycine since it was almost completely hydrolyzed within 3 hr by diluted fecal cultures. Specificity of deconjugating enzymes for either glycine or taurine amidates has been demonstrated in isolated strains (13–15), indicating that there are deconjugating enzymes with different substrate specificity; thus, it is likely that not all fecal cultures will have the same substrate specificity as CGH.

Significance of bacterial deconjugation of bile acid amidates

How well the results of these in vitro studies with pure enzyme or fecal cultures can be used to predict the resistance of these or other cholyl conjugates to bacterial deconjugases in vivo is not known. The only bile acid conjugates for which the metabolism of the steroid and amino acid moiety has been compared simultaneously are cholylglycine (5) and cholyltaurine (34). In these studies, the daily fractional turnover rate of the taurine moiety of cholyltaurine during enterohepatic circulation was much lower than that of the glycine moiety of cholylglycine. This was judged then to be due to less deconjugation of the taurine amidates by the gut microflora, an interpretation supported by the present results with CGH.

Whether bacterial bile acid deconjugation has any biological utility is not known. Deoxycholate and lithocholate, which are the final results of deconjugation and dehydroxylation of the primary conjugated bile acids, are far less soluble at fecal pH than their conjugated primary bile acid precursors (25, 35–37). Thus, deconjugation, which greatly facilitates subsequent 7-dehydroxylation (5, 38, 39), leads to precipitation of these bile acids which otherwise in high aqueous concentrations might well inhibit electrolyte and water absorption in the colon (25, 36, 37).

From a pharmacological standpoint, these results, as well as the report of Batta et al. (32), suggest that it should be possible to synthesize bile acid conjugates that are deconjugated less rapidly, either by using an amino acid which causes steric hindrance near the peptide bond, by N-methylation of the amide bond, or by altering the length of the side chain. For example, Whitney and Vessey (40) have recently reported that C_{23} nor-cholyltaurine is resistant to hydrolysis by CGH. Indeed, Kimura et al. (41) have recently shown that a single dose

of ursodeoxycholylsarcosine is excreted in feces largely in amidated form, indicating that this compound is resistant to bacterial deconjugation in vitro. However, whether this or any other novel bile acid derivative, when administered chronically, would remain resistant to bacterial deconjugation or would induce deconjugating enzymes in the gut microflora specific for the novel conjugate is not known. Nor is it known whether any such novel conjugate, even if permanently resistant to bacterial deconjugation, would have useful therapeutic properties.

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